The Journal of Nutrition
Nutrition and Disease

Inactivation of Adenomatous Polyposis Coli Reduces Bile Acid/Farnesoid X Receptor Expression through *Fxr* gene CpG Methylation in Mouse Colon Tumors and Human Colon Cancer Cells[1–3]

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Abstract

Background: The farnesoid X receptor (FXR) regulates bile acid (BA) metabolism and possesses tumor suppressor functions. FXR expression is reduced in colorectal tumors of subjects carrying inactivated adenomatous polyposis coli (*APC*). Identifying the mechanisms responsible for this reduction may offer new molecular targets for colon cancer prevention.

Objective: We investigated how *APC* inactivation influences the regulation of *FXR* expression in colonic mucosal cells. We hypothesized that *APC* inactivation would epigenetically repress nuclear receptor subfamily 1, group H, member 4 (*FXR* gene name) expression through increased CpG methylation.

Methods: Normal proximal colonic mucosa and normal-appearing adjacent colonic mucosa and colon tumors were collected from wild-type C57BL/6J and *Apc*-deficient ($Apc^{Min/+}$) male mice, respectively. The expression of *Fxr*, ileal bile acid-binding protein (*Ibabp*), small heterodimer partner (*Shp*), and cyclooxygenase-2 (*Cox-2*) were determined by real-time polymerase chain reaction. In both normal and adjacent colonic mucosa and colon tumors, we measured CpG methylation of *Fxr* in bisulfonated genomic DNA. In vitro, we measured the impact of *APC* inactivation and deoxycholic acid (DCA) treatment on *FXR* expression in human colon cancer HCT-116 cells transfected with silencing RNA for *APC* and HT-29 cells carrying inactivated *APC*.

Results: In $Apc^{Min/+}$ mice, constitutive CpG methylation of the *Fxrα3/4* promoter was linked to reduced (60–90%) baseline *Fxr*, *Ibabp*, and *Shp* and increased *Cox-2* expression in apparently normal adjacent mucosa and colon tumors. *Apc* knockdown in HCT-116 cells increased cellular myelocytomatosis *(c-MYC)* and lowered (~50%) *FXR* expression, which was further reduced (~80%) by DCA. In human HCT-116 but not HT-29 colon cancer cells, DCA induced *FXR* expression and lowered CpG methylation of *FXR*.

Conclusions: We conclude that the loss of APC function favors the silencing of FXR expression through CpG hypermethylation in mouse colonic mucosa and human colon cells, leading to reduced expression of downstream targets (SHP, IBABP) involved in BA homeostasis while increasing the expression of factors (COX-2, c-MYC) that contribute to inflammation and colon cancer. *J Nutr* 2016;146:236–42.

Keywords: farnesoid X receptor, bile acid metabolism, adenomatous polyposis coli, deoxycholic acid, epigenetics, CpG methylation, inflammation, colon cancer

Introduction

The farnesoid X receptor (FXR)[7] regulates bile acid (BA) homeostasis through the enterohepatic circulation. In the intestine, FXR activates the expression of ileal bile acid-binding protein (IBABP) and small heterodimer partner (SHP). In turn, SHP represses the intestinal expression of the sodium-dependent BA transporter. In the liver, FXR induces SHP expression, which

[1] Supported by a pilot project (PML and DFR) funded by the University of Arizona Cancer Center support grant P30CA23074.
[2] Author disclosures: OI Selmin, C Fang, AM Lyon, TC Doetschman, PA Thompson, JD Martinez, JW Smith, PM Lance, and DF Romagnolo, no conflicts of interest.
[3] Supplemental Table 1 is available from the ''Online Supporting Material'' link in the online posting of this article and from the same link in the online table of contents at http://jn.nutrition.org.
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[7] Abbreviations used: *APC*, adenomatous polyposis coli; BA, bile acid; c-MYC, cellular myelocytomatosis; *Cox-2*, cyclooxygenase-2; DCA, deoxycholic acid; FAP, familial adenomatous polyposis; FXR, farnesoid X receptor; HFD, high-fat diet; IBABP, ileal bile acid-binding protein; MSP, methylation-specific primer; NT, nontumor; SHP, small heterodimer partner; si*APC*, silencing RNA for adenomatous polyposis coli; Wnt, wingless-type mouse mammary tumor virus integration site family.

Manuscript received April 29, 2015. Initial review completed May 30, 2015. Revision accepted November 3, 2015.
First published online November 25, 2015; doi:10.3945/jn.115.216580.

then inhibits the expression of the cytochrome P450 A1 enzyme that catalyzes the de novo synthesis of BA from cholesterol (1). In human colorectal neoplasms, FXR expression becomes repressed at the late adenoma stage during the transition to carcinoma (2) and is inversely correlated with the grade of malignancy and poor clinical outcome (3). In $Fxr^{-/-}$ knockout mice, the loss of FXR function increases susceptibility to chemically induced colon tumorigenesis (4). Conversely, *Fxr* transgene overexpression in intestinal cells reduces tumor growth (5). Thus, conditions that interfere with normal intestinal cell FXR expression and signaling may compromise normal BA homeostasis, leading to the increased production of a tumor-promoting secondary BA such as deoxycholic acid (DCA) (6–10).

Intestinal expression of FXR is substantially reduced in patients with autosomal dominantly inherited familial adenomatous polyposis (FAP) (5), which is caused by germline mutations in the adenomatous polyposis coli (*APC*) gene. Somatic *APC* mutations occur early in colorectal tumorigenesis (11). Patients with FAP develop numerous colorectal adenomas in their first 2 decades of life that inevitably progress to colorectal cancer unless prophylactic panproctocolectomy is performed (12). *Fxr* expression is reduced in the $Apc^{Min/+}$ mouse (4), which carries an inactivating mutation in the *Apc* gene. The APC protein sequesters β-catenin in the cytosol, thereby preventing the activation of the protumorigenic wingless-type mouse mammary tumor virus integration site family (Wnt) signaling pathway (13, 14). When APC expression is reduced, β-catenin has been shown to translocate to the nucleus and induce members of the transcription factor/lymphoid enhancer-binding factor to form transcription complexes, which in turn increase the expression of protumorigenic cyclin D1 (15, 16) and cellular myelocytomatosis (c-MYC) (17). Although these downstream effects are understood, the initial mechanisms that link *APC* inactivation to reduced FXR expression remain largely unknown.

Our first objective in this study was to investigate the mechanisms that link the inactivation of *Apc* to reduced nuclear receptor subfamily 1, group H, member 4 (*Fxr* gene name) expression in colon tumors. We used the $Apc^{Min/+}$ mouse because it carries mutated *Apc* (18) and is regarded as a good model of multistage colon carcinogenesis (19). We extended these studies to in vitro experiments with human colon cancer cells (HCT-116) carrying wild-type *APC* (20) and transfected with silencing RNA for *APC* (si*APC*) or human colon cancer cells (HT-29) harboring inactivated *APC* (20) and treated with DCA. We selected DCA as a prototype secondary BA because its fecal excretion increases with high-fat diet (HFD) consumption (21, 22), and the accumulation of DCA has been linked to an increased risk of polyps and colorectal tumors (6, 7).

Methods

Mice models. Control C57/BL6J male mice were purchased from Jackson Laboratories. Mice were killed at 12 wk of age, and colonic tissue was isolated for further analyses as described previously (23). Briefly, the large bowel was cut open longitudinally along the main axis and washed with ice-cold PBS. Proximal colonic mucosa was scraped, and colonic cells were separated by centrifugation. $Fxr\alpha^{-/-}$ mice in pure C57BL/6J background were gifts from Frank Gonzalez (Laboratory of Metabolism, National Cancer Institute). C57BL/6J-$Apc^{Min/+}$ mice were purchased from Jackson Laboratories. Female $Fxr\alpha^{-/-}$ mice were crossed with male $Apc^{Min/+}$ to produce $Fxr\alpha^{+/-}$ $Apc^{Min/+}$ mice. Female $Fxr\alpha^{+/-}$ were then crossed with male $Fxr\alpha^{+/-}$ $Apc^{Min/+}$ mice to produce $Fxr\alpha^{-/-}$ $Apc^{Min/+}$, $Fxr\alpha^{+/-}$ $Apc^{Min/+}$, and $Apc^{Min/+}$ genotypes. All genotypes were viable and fertile. All animal procedures with *Fxr* and *Apc* genotypes were approved by the Institutional Animal Care and Use Committee of the Burnham Institute for Medical Research and the University of Arizona. *FXRα* genotyping of tail DNA was performed using PCR as described previously (24). $Apc^{Min/+}$ genotyping was conducted following the protocol from Jackson Laboratories. Mice were housed in conventional cages under a 12-h light/dark cycle with free access to Teklad global rodent diet (Harlan Laboratories) and tap water. The mice were killed by CO_2 asphyxiation at 12 wk of age. Colon tumors and adjacent (1 cm away from the tumor site), normal-appearing colon tissue were collected, rinsed with PBS, and stored at −80°C until further analysis.

Tissue culture and reagents. Human HCT-116 and HT-29 colon cancer cells were obtained from American Type Culture Collection and maintained in DMEM from Sigma-Aldrich supplemented with 10% FBS (Hyclone Laboratories) as described previously (25). At the end of the treatment periods, cells were washed with PBS, harvested, and stored at −80°C until further analysis. DCA was purchased from Sigma-Aldrich.

qRT-PCR. Total RNA was extracted from mucosa scraped from the proximal colon according to the protocol described previously (26) and purified using the Quick-RNA MiniPrep kit according to the manufacturer's instructions (Zymo Research). The concentration and quality of RNA were verified using the Thermo Scientific NanoDrop1000 spectrophotometer. Equal amounts of total RNA (500 ng) were transcribed into cDNA using qScript cDNA SuperMix (Quanta Biosciences). PCR products were amplified from the cDNA fragments using PerfeCTa SYBR Green FastMix, ROX (Quanta Biosciences). Briefly, reactions were run at a final volume of 10 μL consisting of the following master mix: 5 μL of SYBR Green FastMix, 1 μL each of forward and reverse primers (10 nmol/L), 2 μL of nuclease-free water, and 1 μL of cDNA. Amplification of *Gapdh* (*GAPDH* for HCT-116 and HT-29*)* was used for normalizing mRNA expression. The mouse and human primers (Sigma-Aldrich) used for qRT-PCR are shown in **Supplemental Table 1**.

Western blotting and silencing RNA experiments. Immunodetection by Western blotting was performed using antibodies obtained from Santa Cruz Biotechnology (H-130 FXR, β-ACTIN) and EMD Millipore (c-MYC anti-phospho Thr58/Sr62). Silencing RNA experiments were carried out according to the manufacturer's instructions (Dharmacon) as described previously (27). Briefly, 5×10^5 HCT-116 cells were plated in 6-well plates and transfected using the DharmaFECT 2 transfection reagent with nontargeting pool and smart pool human si*APC* for 48 h. Cells were then cultured in control DMEM or DMEM supplemented with 50 μmol/L DCA for an additional 72 h. At the end of the incubation period, cells were harvested for qRT-PCR and Western blotting analyses.

CpG methylation. Promoter methylation was analyzed as described previously (28). Briefly, genomic DNA was isolated from 10–15 mg of proximal colon mucosa using the DNeasy Blood & Tissue Kit (Qiagen). Genomic DNA (1 μg) was subjected to bisulfite modification using the EpiTect Bisulfite Conversion Kit (Qiagen). In preliminary experiments, we verified that the number of cycles for semiquantitatively amplifying each promoter fragment with methylation-specific primers (MSPs) was in the linear range. The bisulfite-modified DNA was analyzed by PCR as follows: 1 cycle at 94°C for 1 min; 35 cycles at 94°C for 30 s, 59°C for 30 s, and 72°C for 1 min; and 1 cycle at 72°C for 5 min. Reactions were carried out at a final volume of 25 μL consisting of the following master mix: 50 ng of bisulfite-modified genomic DNA, 0.4 μL of JumpStart Taq DNA polymerase (Sigma-Aldrich), 2.5 μL of 10× PCR buffer, 3.5 μL of 25 mM $MgCl_2$ (final concentration: 3.5 mmol/L), 0.5 μL of 10 mmol/L deoxyribonucleotide triphosphate mix (final concentration: 200 μmol/L), 1 μL each of forward and reverse primers, and water to bring the final volume to 25 μL. The PCR amplification products were separated on 2% agarose gels and visualized using ethidium bromide staining. PCR amplicons were of the expected size, and their authenticity was confirmed by direct sequencing. The primers (Sigma-Aldrich) used for DNA methylation studies are shown in Supplemental Table 1.

Statistical analysis. Densitometries of CpG-methylated *FXR* after PCR amplification and FXR protein after Western blotting of samples from

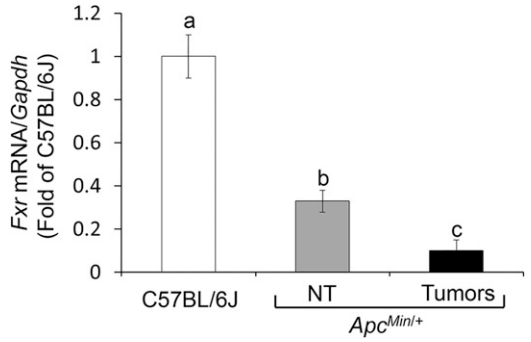

FIGURE 1 *Apc* inactivation reduces *Fxr* expression in adjacent colonic mucosa and colon tumors of $Apc^{Min/+}$ mice. Bars represent qRT-PCR quantitation (fold of C57BL/6J) of *Fxr*/*Gapdh* in NT colonic mucosa and colon tumors from $Apc^{Min/+}$ mice. Values are means ± SEMs, $n = 4$ (mean of 6 replicates/mouse). Means without a common letter differ, $P < 0.05$. *Apc*, adenomatous polyposis coli; *Fxr*, farnesoid X receptor; NT, nontumor.

HCT-116 and HT-29 cells were performed using Kodak ID image EDAS 290 analysis software (Eastman Kodak). Statistical analyses of CpG methylation and expression data from mice and cell culture studies were performed by 1-factor ANOVA after assessing data normality using a Shapiro–Wilk test and variance homogeneity using Bartlett's test. After main effects were found to be significant at $P \leq 0.05$, post hoc multiple comparisons among all means were conducted using Tukey's test.

Results

Apc deficiency correlates with lower Fxr expression in adjacent colonic mucosa and colon tumors. Intestinal accumulation of BA, hyperlipidemia, and reduced FXR expression have been shown to accompany the development of intestinal tumors in *Apc*-deficient models (4, 29). To investigate the mechanisms that link *Apc* deficiency to reduced FXR expression, we first compared mRNA levels in nontumor (NT) proximal colonic mucosa of $Apc^{Min/+}$ mice to those of control wild-type C57BL/6J mice. *Fxr* expression was 60% lower in NT mucosa from the $Apc^{Min/+}$ mice than in colonic mucosa from control mice and was reduced an additional 30% in colon tumors of $Apc^{Min/+}$ mice (**Figure 1**).

Constitutive CpG methylation of Fxrα3/4 promoter in Apc-deficient adjacent colonic mucosa and colon tumors. To determine whether epigenetic mechanisms contributed to reducing *Fxr* expression in the colonic mucosa and tumors of $Apc^{Min/+}$ mice, we studied changes in *Fxrα3/4* CpG promoter methylation (**Figure 2**A, B). We selected the promoter region on exon-3 because it generates *Fxrα3/4* transcripts, which are expressed in the intestine at higher levels compared with the *Fxrα1/2* isoforms transcribed from exon-1 (1). In FAP patients, *FXRα3/4* transcripts are markedly reduced compared with *FXRα1/2* variants (30). In preliminary experiments, we confirmed that amplification with MSPs of *Fxrα3/4* promoter fragments occurred in the linear range (Figure 2C). Accounting for the reduction in *Fxr* expression, CpG methylation of the *Fxrα3/4* promoter was ~0.4-fold higher in NT mucosa and ~0.8-fold higher in $Apc^{Min/+}$ mouse colonic tumors compared with levels found in wild-type C57BL/6J mice (**Figure 3**A). Using MSPs, we also performed direct-sequence analyses of bisulfonated genomic DNA obtained from 4 independent DNA clones derived from 4 separate $Apc^{Min/+}$ tumors. We found that 13 CpG sites flanking the transcription start site on exon-3 (Figure 3B) were consistently methylated.

Apc deficiency affects the expression of FXR target genes in adjacent colonic mucosa and colon tumors. The *Shp* and *Ibabp* genes are putative FXR transcriptional targets (1). Compared with wild-type C57BL/6J mice, *Shp* (**Figure 4**A) and *Ibabp* (Figure 4B) levels were reduced by 90–95% in $Apc^{Min/+}$ NT mucosa and colon tumors. Conversely, cyclooxygenase-2 (*Cox-2*) expression (Figure 4C) in NT colon mucosa and tumors from $Apc^{Min/+}$ compared with wild-type C57BL/6J mice was increased ~0.9- and ~2.5-fold, respectively. As a positive control, we found similar patterns of reduced *Shp* (Figure 4A) and *Ibabp* (Figure 4B) and increased *Cox-2* (Figure 4C) expression in NT mucosa and colon tumors obtained from $Apc^{Min/+}$ $Fxr^{-/-}$ double knockout mice. Taken together, these animal data indicate that baseline *Fxr* expression was reduced in *Apc*-deficient colon cells and tumors in accordance with constitutive CpG methylation of the *Fxrα3/4* promoter. Silencing of *Fxr* was associated with downregulation of FXR target genes (*Shp* and *Ibabp)* and upregulation of proinflammatory *Cox-2*.

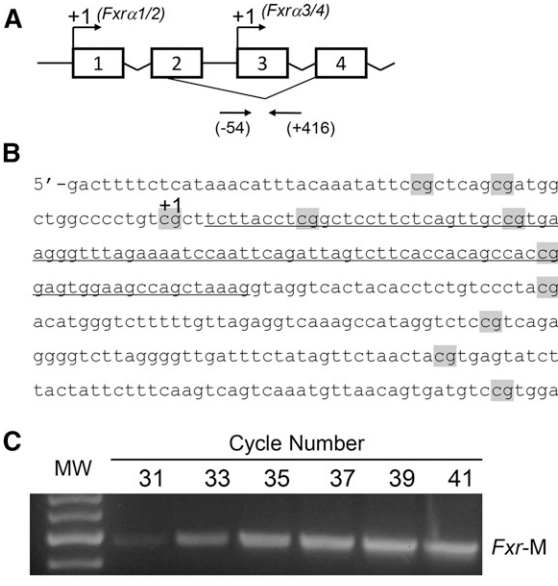

FIGURE 2 Organization of the mouse nuclear receptor subfamily 1, group H, member 4 (*Fxr* gene name). (A) Top arrows indicate transcription start sites (+1) on exon-1 and -3. Bottom arrows indicate positions of oligonucleotides (−54/+416) around exon-3 used for *Fxr* CpG methylation studies. (B) Nucleotide sequence flanking exon-3. CpGs are highlighted in gray. The +1 indicates the transcription start site on exon-3. The underlined nucleotide sequence corresponds to exon-3. (C) PCR cycle number and MW control for amplification of *Fxr*-M. PCR bands were amplified from bisulfonated genomic DNA using mouse methylation-specific primers. *Fxr*, farnesoid X receptor; *Fxr*-M, methylated mouse *Fxrα3/4* promoter; MW, molecular weight.

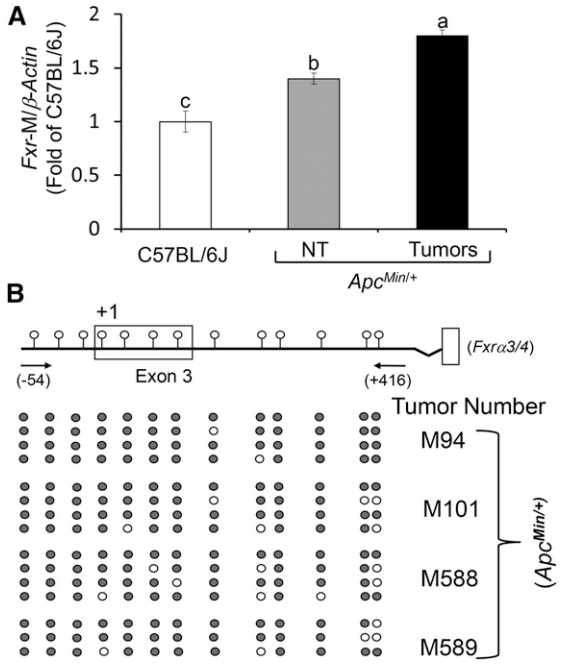

FIGURE 3 *Apc* inactivation correlates with constitutive CpG methylation of *Fxr* in adjacent colonic mucosa and colon tumors of $Apc^{Min/+}$ mice. (A) Bars represent qRT-PCR quantitation (fold of C57BL/6J) using methylation-specific primers of *Fxr*-M/*β-actin* in NT colonic mucosa and colon tumors from $Apc^{Min/+}$ mice. Values are means ± SEMs, $n = 4$ (mean of 6 replicates/mouse). Means without a common letter differ, $P < 0.05$. (B) Position of CpGs in exon-3 of the mouse *Fxr* gene. Black circles indicate methylated and white circles indicate unmethylated CpGs of 4 clones with 10 replicates per clone from 4 independent colon tumors. *Apc*, adenomatous polyposis coli; *Fxr*, farnesoid X receptor; *Fxr*-M, *Fxrα3/4* promoter methylation.

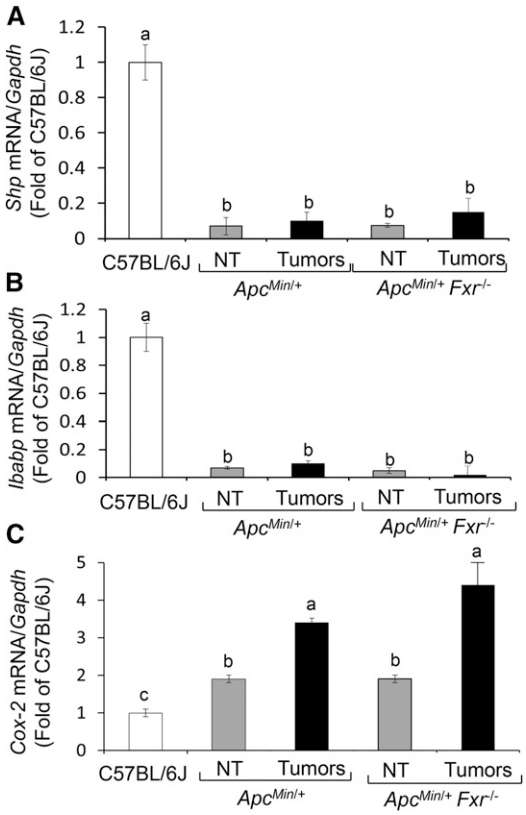

FIGURE 4 *Apc* inactivation leads to constitutive repression of *Shp* and *Ibabp* and activation of *Cox-2* expression in adjacent colonic mucosa and colon tumors of $Apc^{Min/+}$ mice. Bars represent qRT-PCR quantitation (fold of C57BL/6J) for (A) *Shp*, (B) *Ibabp*, and (C) *Cox-2* corrected for *Gapdh* as an internal control in NT colonic mucosa and colon tumors from $Apc^{Min/+}$ and $Apc^{Min/+}$ $Fxr^{-/-}$ mice. Values are means ± SEMs, $n = 4$ (mean of 6 replicates/mouse). Means without a common letter differ, $P < 0.05$. *Apc*, adenomatous polyposis coli; *Cox-2*, cyclooxygenase-2; *Fxr*, farnesoid X receptor; *Ibabp*, ileal bile acid-binding protein; NT, nontumor; *Shp*, small heterodimer partner.

APC deficiency hampers the stimulation of FXR expression by DCA in human colon cancer cells in vitro. To further examine the impact of *APC* inactivation on the regulation of *FXR* expression, we transfected HCT-116 cells with si*APC*. Compared with cells transfected with nontarget silencing RNA, a large (~80%) reduction in *APC* mRNA expression was accompanied by increased levels (~0.4-fold) of *c-MYC* transcripts (**Figure 5**A). c-MYC is a downstream target for the activated Wnt/β-catenin pathway (17). As a positive control for tumor promotion by DCA in *APC*-deficient cells, we observed that the treatment of HT-29 colon cancer cells with DCA induced c-MYC protein levels (~4.0-fold) (Figure 5B). In HCT-116 cells transfected with si*APC*, we observed a ~50% reduction in *FXR* mRNA levels, which were reduced by an additional 30% when exposed to DCA (Figure 5C).

To simulate the exposure of colon cells with wild-type *APC* to secondary BA in vitro, we treated human HCT-116 colon cancer cells with DCA. In response to the DCA treatment, *FXR* expression increased 1.5-fold in HCT-116 cells (**Figure 6**A). This was accompanied by reduced (~50%) *FXR* CpG methylation (Figure 6B) and accumulation (1.0-fold) of the FXR protein (Figure 6C). Conversely, DCA treatment did not elicit significant changes in *FXR* CpG methylation (Figure 6B) and FXR protein (Figure 6C) concentrations in HT-29 cells. Overall, these cumulative in vitro findings suggested that in human colon cells harboring wild-type *APC* the DCA-induced expression of *FXR* was associated with a reduction in *FXR* CpG methylation. Conversely, in *APC*-deficient human colon cancer cells the *FXR* gene was refractory to DCA stimulation in association with increased expression of the c-MYC oncogene.

Discussion

Despite widespread screening and advances in treatment, colorectal cancer remains the second cause of cancer death in the United States (31). The FXR is a transcriptional regulator of several enterohepatic metabolic pathways (1, 32). Importantly, reduced FXR expression is associated with intestinal tumorigenesis in human subjects (3, 5) and animal models (4, 5).

In this study, the first objective was to examine whether changes in *Fxr* CpG methylation contributed to regulating *Fxr* expression in apparently normal colonic mucosa and colon tumors of $Apc^{Min/+}$ mice. The $Apc^{Min/+}$ mouse is a model of human FAP caused by mutations in the *APC* gene (4, 5). We found that the region of the *Fxr* gene flanking the transcription start site harbored in exon-3 (*Fxrα3/4*) was constitutively hypermethylated in nonneoplastic colonic mucosa and to a larger degree in colon tumors of $Apc^{Min/+}$ mice. The increased CpG methylation of the *Fxrα3/4* promoter correlated with the reduced expression of *Shp* and *Ibabp* and accumulation of *Cox-2*. The latter changes were also seen in colon tumors of $Apc^{Min/+}$ $Fxr^{-/-}$ mice, which provided a positive control for the *Fxr* CpG

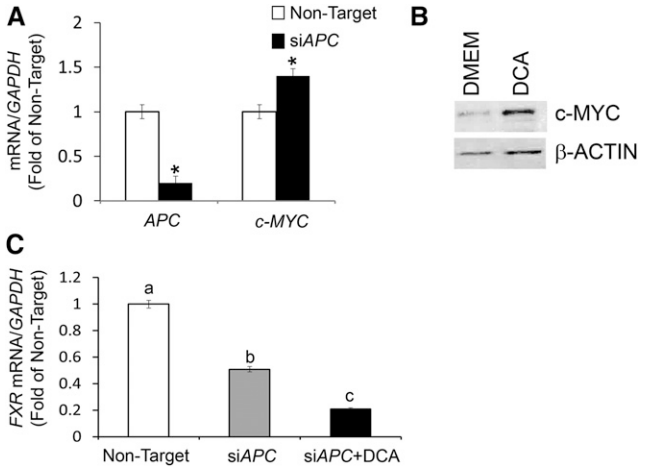

FIGURE 5 *APC* silencing represses expression of *FXR* in human colon cancer cells. (A) Bars represent qRT-PCR quantitation (fold of nontarget) from 2 separate experiments performed in triplicate for *APC* and *c-MYC* corrected for *GAPDH* mRNA as the internal control in human HCT-116 colon cancer cells transfected with nontarget and si*APC*. Values are means ± SEMs. (B) Bands are representative immunocomplexes detected by Western blotting for c-MYC and β-ACTIN in HCT-29 cells cultured in control DMEM or DMEM supplemented with DCA (50 μM for 72 h). (C) Bars represent qRT-PCR quantitation (fold of nontarget) from 2 separate experiments performed in triplicate of *FXR* in human HCT-116 colon cancer cells transfected with nontarget and si*APC* and in the presence of DCA (50 μM for 72 h). Values are means ± SEMs. *Different from nontarget, $P < 0.05$. *APC*, adenomatous polyposis coli; c-MYC, cellular myelocytomatosis; DCA, deoxycholic acid; *FXR*, farnesoid X receptor; si*APC*, silencing RNA for *APC*.

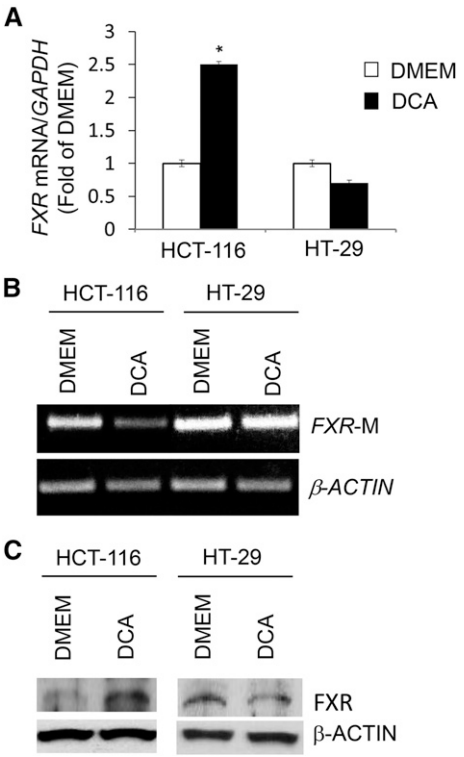

FIGURE 6 Differential regulation of FXR expression by secondary bile DCA in wild-type *APC* and *APC*-deficient human colon cancer cells. (A) Bars represent qRT-PCR quantitation (fold of DMEM) from 2 separate experiments performed in triplicate of *FXR* corrected for *GAPDH* as internal control in human HCT-116 (wild-type *APC*) and HT-29 (inactivated *APC*) colon cancer cells after 72 h of treatment with 50 μM DCA. Values are means ± SEMs. *Different from DMEM, $P < 0.05$. (B) Methylated PCR products were amplified with human *FXR*-M and β-*ACTIN* methylation-specific primers using as a template bisulfonated genomic DNA obtained from HCT-116 and HT-29 colon cancer cells. (C) Bands are representative immunocomplexes detected by Western blotting for FXR and β-ACTIN in HCT-116 and HT-29 cells cultured in control DMEM or DMEM supplemented with DCA. *APC*, adenomatous polyposis coli; DCA, deoxycholic acid; *FXR*, farnesoid X receptor; *FXR*-M, *FXRα3/4* promoter methylation.

methylation and *Shp* and *Ibabp* expression studies. The marked reduction in *Shp* and *Ibabp* expression in both nonneoplastic colonic mucosa, and colon tumors of $Apc^{Min/+}$ mice were consistent with the fact both *Shp* and *Ibabp* genes are direct targets for transcriptional activation by FXR (1). Conversely, we attributed the activation of *Cox-2* expression observed in $Apc^{Min/+}$ and $Apc^{Min/+}$ $Fxr^{-/-}$ mice to overriding of the negative feedback by FXR on the NF-κB/Cox-2 axis (33). Therefore, a possible implication of these findings is that changes in CpG methylation in the *Fxr* and *Cox-2* genes could serve as sentinel biomarkers of intestinal inflammation and tumorigenesis associated with *Apc* inactivation. In support of this idea, early changes in CpG methylation profiles of genes involved in lipid metabolism and inflammation are being considered as epigenetic predictors of colon tumor development in humans (34).

The *Fxr* CpG methylation and expression data presented in this study complement those of a recent study (35) that documented reduced *FXR* expression in human precancerous lesions and colon tumors. The authors of the latter study, however, did not observe any changes in *FXR* promoter methylation at a distal CpG island comprising 11 CpGs and spanning the 5′ region upstream (∼−3.2 to −2.9 kb) from the transcription start site of exon-1. We focused our methylation studies on a proximal 470-bp region harboring 13 CpG dinucleotides and flanking the transcription start site of exon-3. Compared with *FXRα1/2* variants, *FXRα3/4* transcripts are markedly reduced in the tumors of FAP patients (30). Therefore, *APC* gene inactivation in intestinal cells may lead to preferential CpG methylation in the proximal *FXRα3/4* promoter. This specificity may be related to the evidence that *FXRα3/4* variants are transcribed at higher amounts in the intestine (1). Moreover, the alternative usage of transcription start sites on the *FXR* gene and expression of *FXR* variants (36, 37) may be related to differential regulation at various stages of colon tumorigenesis (35). Therefore, future studies should compare the effects of *APC* deficiency on CpG methylation and the expression of *FXR* splicing variants at various tumor stages (e.g., adenomas compared with adenocarcinomas) of colon tumor development.

The silencing of *APC* in human HCT-116 colon cancer cells abrogated basal *FXR* expression, which was reduced further upon cotreatment with DCA. These changes culminated with the upregulation of *c-MYC*, a downstream target for the Wnt/β-catenin pathway (17). In nonneoplastic mucosa of the ileum and proximal colon, *APC* and *FXR* expression has been shown to follow an increasing gradient from the bottom of the crypts to the top of the intestinal villi and luminal surface of the colonic crypts (3, 38). These expression patterns combined with published (4, 5) and our data suggest that APC may be required for properly regulating *FXR* expression and the differentiation of colonic cells (39). In support of this idea, we observed that the in vitro treatment of human HCT-116 colon cancer cells with physiological concentrations of DCA (40), a prototype secondary BA, induced *FXR* expression and reduced *FXR* CpG

methylation. Although HCT-116 cells have been found to harbor 1 mutated allele for the catenin (cadherin-associated protein), β1, 88-kDa gene that encodes β-catenin, APC/β-catenin complexes are functional in these cells (20). In contrast, in human HT-29 colon cancer cells that harbor a mutated *APC* gene, modest changes in *FXR* expression or CpG methylation were observed in response to treatment with DCA, which, however, induced c-MYC expression, a downstream target of the Wnt/β-catenin pathway. These data are in accordance with those of earlier studies that reported constitutive activation of the Wnt/β-catenin pathway in the small intestine of $Apc^{Min/+}$ mice (41) and in immune-deficient nude mice xenografted with HT-29 colon cancer cells (42).

Taken together, our mice data show that defective *Apc* expression induces CpG methylation in the *Fxr* gene and compromises the expression of downstream targets (e.g., *Shp*, *Ibabp*) necessary to maintain BA homeostasis. Moreover, reduced *Fxr* expression is accompanied by constitutive accumulation of proinflammatory *Cox-2*. We hypothesize that this environment may reinforce the tumor-promoting effects of secondary BAs such as DCA. The requirement for normal *APC* in activating FXR expression is corroborated by our in vitro observations with human colon cancer cells. Ongoing studies in our laboratory are exploring the signaling cascades through which *APC* inactivation modulates the placement of CpG methylation and other epigenetic marks (e.g., histone modifications) on the *FXR* gene and the potential effects of reduced FXR expression on the microbiome and production of secondary BAs (43). An important question raised by the current observations is whether individuals who carry mutated (familial) or inactivated (sporadic) APC and adhere to a HFD may be at higher risk of developing colon inflammation and cancer induced by secondary BAs via reduced FXR expression. Therefore, future studies should examine the differential effects of various HFDs (e.g., n–6 compared with n–3) on *FXR* CpG methylation in normal and *APC*-deficient colon models. A second question pertains to whether other epigenetic regulatory defects associate with tumor development in APC-deficient colon cells. Recent studies conducted in the $Apc^{Min/+}$ model provided evidence that tumor-related hypermethylation appeared as a progressive event and reached higher levels in advanced tumor stages (44, 45). Consequently, progress in understanding the mechanisms responsible for DNA methylation dynamics in the *Fxr* and other genes may unravel how interactions between the inactivation of *APC* and exposure to dietary FAs influence the risk of inflammatory bowel diseases (46, 47) and colorectal tumors (42, 48) and thus offer new epigenetic targets for diagnosis and prevention.

Acknowledgments

OIS and AML conducted the research and analyzed the data; CF and JWS provided the $Apc^{Min/+}$ and $Apc^{Min/+}$ $Fxr^{-/-}$ control and tumor samples; OIS, PML, and DFR wrote the manuscript; and DFR had primary responsibility for final content. All authors contributed to project conception and have read and approved the final manuscript.

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